THE USE OF ELEMENTAL MASS SPECTROMETRY IN PHOSPHOPROTEOMIC APPLICATIONS

Evelyne Maes,[1,2]* Kristof Tirez,[1] Geert Baggerman,[1,3] Dirk Valkenborg,[1,3,4] Liliane Schoofs,[2] Jorge Ruiz Encinar,[5] and Inge Mertens[1,3]

[1]*Flemish Institute for Technological Research (VITO), Mol, Belgium*
[2]*KU Leuven, Research Group of Functional Genomics and Proteomics, Leuven, Belgium*
[3]*CFP-CeProMa, University of Antwerp, Antwerp, Belgium*
[4]*Interuniversity Institute for Biostatistics and Statistical Bioinformatics, Hasselt University, Diepenbeek, Belgium*
[5]*Department of Physical and Analytical Chemistry, University of Oviedo, Oviedo, Spain*

Received 11 December 2013; revised 14 May 2014; accepted 14 May 2014

Published online in Wiley Online Library (wileyonlinelibrary.com). DOI 10.1002/mas.21440

Reversible phosphorylation is one of the most important post-translational modifications in mammalian cells. Because this molecular switch is an important mechanism that diversifies and regulates proteins in cellular processes, knowledge about the extent and quantity of phosphorylation is very important to understand the complex cellular interplay. Although phosphoproteomics strategies are applied worldwide, they mainly include only molecular mass spectrometry (like MALDI or ESI)-based experiments. Although identification and relative quantification of phosphopeptides is straightforward with these techniques, absolute quantification is more complex and usually requires for specific isotopically phosphopeptide standards. However, the use of elemental mass spectrometry, and in particular inductively coupled plasma mass spectrometry (ICP-MS), in phosphoproteomics-based experiments, allow one to absolutely quantify phosphopeptides. Here, these phosphoproteomic applications with ICP-MS as elemental detector are reviewed. Pioneering work and recent developments in the field are both described. Additionally, the advantage of the parallel use of molecular and elemental mass spectrometry is stressed. © 2014 Wiley Periodicals, Inc. Mass Spec Rev

Keywords: *phosphoproteomics; phosphorylation; ICP-MS; ^{31}P; absolute quantification*

I. INTRODUCTION

Unraveling molecular pathways in order to understand cellular processes in biological systems is still a major challenge. Because the development of soft-ionization mass spectrometric proteomic applications made it possible to identify and quantify proteins in biological systems in a high-throughput way, major breakthroughs were achieved (Aebersold et al., 2013). The field of proteomics, with procedures linked to soft-ion sources like matrix assisted laser desorption/ionization mass spectrometry (MALDI-MS) and electrospray (ESI)-MS, is expanding with more and more applications in bottom-up, shotgun, and top-down strategies and quantitative label-based or label-free approaches (Zhang et al., 2013). In these proteomic applications, molecular MS is applied to identify peptides and proteins or to obtain structural information about them. Despite the unquestionable success of soft-ionization sources, some research domains essential to understand the role and function of biomolecules are still underexploited (Mounicou, Szpunar, & Lobinski, 2010).

One of these research areas is the investigation of post-translational modifications (PTMs). Because these modifications are important mechanisms that diversify and regulate proteins in cellular processes, knowledge about their extent and quantity is very important to understand the complex cellular interplay (Zhao & Jensen, 2009). One of the most important reversible PTM in mammalian cells is phosphorylation. Protein phosphorylation is a key effector of cellular life that regulates metabolism, homeostasis, signal transduction, proliferation, differentiation, and cell survival (Thingholm, Jensen, & Larsen, 2009). Two enzyme groups, kinases and phosphatases, maintain the spatial and temporal control of the phosphorylation and dephosphorylation events, respectively (Paradela & Albar, 2008). The importance of this PTM is reflected by the fact that up to one third of the proteins in a regular mammalian cell are covalently bound to a phosphate group during their lifetime (Paradela & Albar, 2008). Also, abnormal phosphorylation is very often a cause or consequence of many (human) diseases. Because only a small percentage of proteins present in a sample are phosphorylated at a definite time, the study of (de) phosphorylation events in a complex biological sample, even with the current optimized enrichment procedures used, remains a huge and challenging task (Nilsson, 2012).

Today, most phosphoproteomics research is based on molecular mass spectrometry (Roux & Thibault, 2013). However, these soft-ionization techniques have limitations because there is a strong dependence on peptide ionization efficiency due to presence of concomitant molecules or the influence of the sample matrix and the limited linear dynamic range. In phosphoproteomics specifically, ionization of phosphopeptides

Contract grant sponsor: Stichting Emmanuel van der Schueren grant from the Vlaamse Liga tegen kanker (VLK).
Correspondence to: Evelyne Maes, Flemish Institute for Technological Research (VITO), Boeretang 200, 2400 Mol, Belgium.
E-mail: evelyne.maes@vito.be

in ESI-MS or MALDI-MS is hindered by suppression effects that make phosphopeptide detection not straightforward (Leitner, Sturm, & Linder, 2011). Moreover, in molecular MS, peptide/protein quantitation strategies are mostly relative approaches. With, for example, peptides with incorporated stable isotopes, fold-change differences in protein abundance of different experimental groups can be obtained, because these labeled peptides are expected to ionize with the same efficiency as their unlabeled counterparts. However, no information on the absolute quantity of the proteins is supplied. It is thus impossible to make quantitative results either traceable to a certified standard or comparable to other results obtained in different conditions/time. Absolute quantitation of (phospho)proteins is thus only feasible when synthetically produced protein standards are available (Kosako & Nagano, 2011). However, these peptide standards for absolute quantification are very rare.

Although often not mentioned in reviews about phosphoproteomics, elemental mass spectrometry can also be applied in this context. In contrast to molecular MS techniques, where molecular information is achieved, elemental mass spectrometry uses hard-ionization sources to obtain only elemental information (Sanz-Medel et al., 2008). For these phosphoproteomics applications, elemental mass spectrometry and in particular inductively coupled plasma—mass spectrometry (ICP-MS) appears advantageous. The use of ICP-MS is possible in proteomic studies, as long as an ICP-detectable heteroatom is present in the proteins. This approach makes it thus conceivable to study, for example, metal-biomolecule interactions or to absolutely quantify proteins/peptides that carry heteroatoms (Bettmer et al., 2009). More importantly, with ICP-MS, absolute quantification of some post-translational modifications in biomolecules that contain biologically important heteroatoms covalently attached to the protein, like phosphorylation, iodination, or metalation can be performed (Mounicou, Szpunar, & Lobinski, 2010). Due to the tremendous instrumental developments during the last decade in the field of ICP-MS, growing interest exists in the properties of this analytical technology as element-specific detector (Pröfrock & Prange, 2012). Nevertheless, successful application of such "elemental" approaches depends on the complementary use of molecule-specific detection techniques such as MALDI-MS or ESI-MS to elucidate amino acid sequences of the peptides/proteins detected with ICP-MS and to gain the necessary information related to stoichiometry (Becker & Jakubowski, 2009; Bettmer et al., 2009; Pröfrock & Prange, 2012). In fact, even though ICP-MS is, at present, mostly applied for fast multi-element determination in environmental research, the technique has a clear potential in proteomics research as well.

Although MALDI-MS and ESI-MS are already accepted in life science proteomic studies, new ICP-MS applications are pending. In this review, we will show how ICP-MS can complement the information obtained from molecular mass spectrometry in phosphoproteomics applications. The use of these integrated multi-technique MS approaches offers the possibility to obtain insights in biological processes in a short period of time. In addition, ICP-MS can provide fast phosphopeptide screening options in complex samples and allows quantification of phosphoproteins in absolute terms. This absolute quantitative determination of phosphorylation will deliver valuable data on signal transduction/regulatory pathways and networks based on phosphorylation.

II. ELEMENTAL MASS SPECTROMETRY

Elemental mass spectrometry, sometimes called "atomic" or "inorganic" mass spectrometry, is an important analytical technique to determine element concentration. Although glow discharge plasma–MS, microwave-induced plasma-MS, and direct current plasma–MS have several applications in environmental studies, it was the development of ICP-MS that boosted the "elemental" mass spectrometry to new applications in the field (Ray et al., 2004). Nowadays, ICP-MS is the most popular element-specific detection technique for fast multi-element determination at the sub-ppb concentration level in life sciences and environmental research (Lobinski, Schaumloffel, & Lobinski, 2006; Pröfrock & Prange, 2012). ICP-MS is also the base of most of the elemental speciation methodologies developed so far in these fields (i.e., organotin, Cr III-VI, organomercury, selenoproteomics).

Since the introduction of the first commercial ICP-MS instrument in 1983, the technique has expanded considerably, with more than 5,000 instruments sold worldwide. Part of this success is due to the great advantage of the species- and matrix-independent signals in ICP-MS, which result in the possibility of generic quantifications without the need for specific standards for each compound (Siethoff et al., 1999). This characteristic enabled also post-column addition of enriched stable isotopes to quantify compounds for which standards were not available. In addition, the great (bio)analytical potential of ICP-MS is the possibility to determine non-metals (e.g., P, S, I, Br), metalloids (Se, As), and metals (Fe, Cu, Zn, Cd, etc.) associated with important biomolecules with extreme sensitivity and specificity (Sanz-Medel, 2010).

A. The Principle of ICP-MS

In inductively coupled plasma mass spectrometers, the ionization source is a plasma of an ionized noble gas, mostly argon, which is generated in a torch by means of an intense electromagnetic field (Sanz-Medel et al., 2008; Bettmer et al., 2009; Wang et al., 2010). With this plasma discharge, a temperature of about 8,000˚C is reached. This high plasma temperature allows an efficient vaporization of the sample, mostly introduced as a liquid form into the introduction system. The sample reaches the base of the plasma through an injection channel of the ICP torch. Here, the sample is successfully dried, followed by dissociation, atomization, excitation, and finally ionization of all sample molecules, independent of the structure of the original substance. In the resulting gas, almost 100% of the produced ions are singly charged. Next, an efficient ion extraction, ion transportation through the mass analyzer, and detection take place. The resulting mass spectrum represents a graph of relative abundances of ions as a function of mass-to-charge (m/z) ratio (mass range = 5–250 Da). Some overlap between isotopes occurs, but for most of the elements, an isotope free of interferences can be found (Wang et al., 2010).

In principle, most of the elements in the periodic table can be detected with ICP-MS, but some elements will have limitations due to high blank values due to entraining air into the ICP-MS source or the solvents used to dissolve samples or high ionization potentials (>11 eV). This first group of elements that cannot be determined with ICP-MS are C, H, O, N, and F. A

second group of elements experiences some major spectral polyatomic interferences due to cluster formation of the most-abundant ions in the plasma such as carbon, nitrogen, oxygen and argon. This group includes, among others, P, S, Se, Ca, and Fe, which are of enormous interest in biological and life science applications. However, this limitation is surmounted by the application of interference-reducing instruments. For many years, quadrupole ICP-MS represents the most frequently used instrumentation as element detector. However, due to the presence of such polyatomic interferences, which are naturally formed in argon plasma under normal laboratory conditions, accurate multi-element determination with regular quadrupole instruments remains challenging.

These interference problems have been overcome by the availability of high-resolution instruments like sector-field (SF) ICP-MS or collision/reaction cell technologies. SF-ICP-MS can be operated in a high-resolution mode ($\Delta m/m \sim 10{,}000$), which allows most target elements to be resolved from interfering polyatomic ions (Becker & Dietze, 1998). However, this enhanced mass resolution results in a reduced ion transmission and, as a consequence, in a strong loss of sensitivity. To counter such loss of sensitivity, quadrupole ICP instruments were developed with an integrated collision/reaction cell. With cell gases such as H_2, He, O_2, or NH_3, most polyatomic interferences can be physically/chemically separated from targeted ions or minimized to an insignificant level with different gas-phase mechanisms. However, application of the reaction cell technology is often at the cost of the multi-elemental capabilities of the quadrupole instrument because the reaction gas in the cell will significantly reduce the sensitivity of other elements. Whenever precise isotope ratio measurements are important, multicollector sector-field ICP-MS can be applied. This high-resolution instrument allows one to detect and measure multiple isotopes at exactly the same time with multiple detectors (Moldovan et al., 2004).

Nowadays, even more advanced mass spectrometric instruments like ICP-QQQ-MS are implemented in elemental research applications (Diez et al., 2012). Here, two independent quadrupole mass filters are connected via an octopole collision/reaction cell. This configuration results in improved reduction of interference and better control of gas-phase reactions.

The main advantage of ICP-MS instruments is its high sensitivity that leads to detection limits of 1–10 femtograms for most elements and even subfemtogram levels for rare earth elements (Wang et al., 2010). Only for elements that are very hard to ionize with high ionization potentials (above 8 eV) like S, Se, and P, the femtogram detection limit cannot be reached. These good detection limits, accompanied by the high sensitivity of ICP-MS, allows simultaneous measurement of all the elements with a linear dynamic range that exceeds 9 orders of magnitude. Furthermore, the high plasma temperature ensures a complete fragmentation of every sample molecule introduced into the ICP-MS that leads to loss of all molecule-specific information, and leaves only their detectable elements. A major advantage of these element detectors is that this near independence of sensitivity towards structure and chemical environment of the target molecules makes it possible to perform species-independent calibration strategies if nebulization efficiencies of the different element-containing species involved are very similar. This characteristic is a main advantage of ICP-MS in contrast to molecular mass spectrometry. Thus, for quantification purposes, only simple elemental standards are required even if metal-, P-, S- or Se-containing species in biological systems must be determined (Svantesson, Pettersson, & Markides, 2002; Sanz-Medel, Montes-Bayon, Luisa Fernandez, 2003; Sanz-Medel et al., 2008; Bettmer et al., 2009; Sanz-Medel, 2010; Wang et al., 2010). Other attractive features of these ultratrace elemental detection instruments are the specificity for accurate detection and quantification and the possibility to obtain precise isotope ratio information.

Also, although ICP-MS only seems interesting as a pure elemental detector, because all molecule-specific information is lost, some molecular specificity ("speciation") can be obtained (Templeton et al., 2000).

B. Hyphenated Techniques

Because ICP-MS produces structure-independent signals, and thus loss of any structural information, a separation of the different element-containing species present in the sample must be made prior to their entrance into the ICP source. Therefore, combination of ICP with separation techniques such as chromatography and electrophoresis allows the elemental detector to obtain some speciation information (Szpunar, Lobinski, & Prange, 2003).

To further characterize heteroatom-containing biomolecules, different couplings have been demonstrated: direct nebulization of liquid chromatographic eluents, laser ablation of proteins after gel electrophoresis, or capillary electrophoresis coupled with ICP-MS. A schematic representation of these hyphenated techniques can be found in Figure 1. The multi-elemental detection capabilities of ICP-MS can thus be exploited to multi-element screening of many heteroatoms in a single experiment.

1. LC-ICP-MS

In the coupling of the multi-isotope character of ICP-MS with separation techniques, high performance liquid chromatography (HPLC) is often preferred. The coupling of LC to ICP-MS is relatively straightforward via a direct connection of the separation column and the nebulizer to allow the direct nebulization of the chromatographic eluent (Pröfrock, 2010). Moreover, great versatility is achieved due to the LC separation characteristics: separation of very complex mixtures can be achieved as a function of hydrodynamic volumes (size-exclusion), hydrophilic/hydrophobic character (normal and reverse phase), and charge states (anion- and cation-exchange) (Montes-Bayon, DeNicola, & Caruso, 2003).

Coupling of reverse phase (RP) chromatography to ICP-MS, however, often complicates elemental detection capabilities. The use a gradient composed of organic solvents, like acetonitrile, changes a number of fundamental parameters over time such as plasma temperature, electron density, aerosol generation, or analyte transport as well as overall ionization that takes place inside the plasma (Grindlay et al., 2008). Furthermore, these organic solvents can introduce even more detrimental effects in standard LC configurations such as additional plasma instabilities, carbon deposition on the cones and lens system, and signal suppression or enhancements. Although some problem-solving features are proposed, including membrane desolvation, spray chamber temperatures below 0°C,

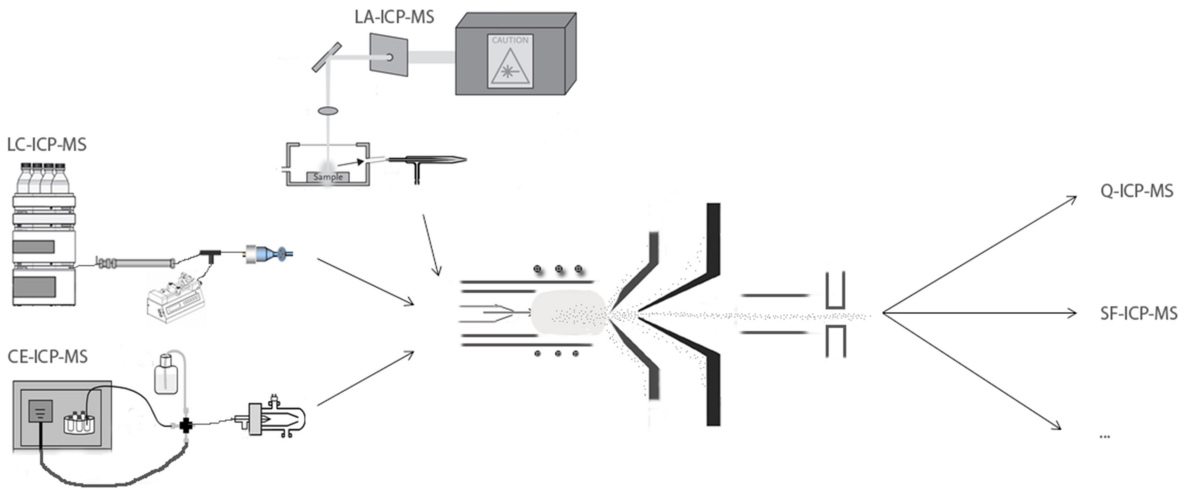

FIGURE 1. Overview of hyphenation techniques to ICP-MS.

addition of an auxiliary flow of O_2 to the plasma, and reduced injector tubing, only miniaturization of the system to capillary or even nano LC seems to work properly (Schaumloffel, Ruiz, & Lobinski, 2003; Schaumloffel, 2007; Pröfrock, 2010).

As in standard proteomics applications, capillary and nano-HPLC show high separation efficiencies combined with low solvent consumption and small sample volumes. However, coupling of these low flow effluents with standard ICP introduction systems was not initially possible. It is only since the development of low flow and total consumption nebulizers that efficient and stable nebulization (evaporation) of flow rates in the range of the low microliter per minute (or even nl/min) is now achievable (Schaumloffel, 2007). In contrast to ESI-MS, ICP-MS is a mass flow-dependent detection technique. In this regard, caution should be applied because the ion signal is proportional to the total number of atoms detected per unit of time (Stefanka et al., 2006). As a result, the improved peak concentration due to application of the most-efficient capillary/nano-LC does not necessarily result in an enhanced response in the ICP-MS system, as observed for ESI-MS.

Due to the successful implementation of capLC and nanoLC-ICP-MS, parallel operation of ICP-MS and ESI-MS under exactly the same chromatographic conditions becomes possible. For proteomics purposes, parallel ICP-MS detection offers a number of advantages with regard to monitor digestion efficiency and quantification of heteroatom-bearing peptides (Bettmer et al., 2009).

2. *LA-ICP-MS*

With solid samples, coupling of laser ablation (LA) to ICP-MS has opened new perspectives to analyze elemental compositions. In laser ablation, a laser beam ablates the sample surface to generate a small cloud of particles and droplets. A significant fraction of the ablated material is carried into a continuous flow of argon to the ICP-MS, where. the sample is ionized and analyzed. The major advantage is the capability to focus and direct a laser precisely, to thereby create a microprobe technique with the capability of spatially resolved sampling (Becker et al., 2007; Becker & Becker, 2010).

Laser ablation-ICP-MS has undergone rapid development in recent years, with even femto laser pulsing applications, to make it applicable for several research fields including biomedical research. Two major applications are laser ablation on gel electrophoresis samples and laser ablation directly on tissues.

The combination of gel electrophoresis and LA-ICP-MS is mostly applied for qualitative and quantitative analysis of different types of heteroatom-containing proteins (Becker et al., 2010; Becker & Becker, 2010). To do so, a critical prerequisite in metal-protein speciation is that the integrity of the heteroatom-protein complex remains intact during the whole sample process, including separation (Konz et al., 2012). Therefore, most LA-ICP-MS studies involve covalently attached heteroatoms, including phosphorus (phosphate moiety in phosphoproteins) or sulfur (methionine and cysteine amino acids). Another approach uses gel electrophoresis in combination with electroblotting onto nitrocellulose or PVDF membranes. That strategy has potential because proteins are concentrated on a thin layer. However, losses during blotting are often mentioned (Konz et al., 2012) and are detrimental when one wants to perform accurate quantitative analysis.

In tissues, visualization of metals, metalloids, and selected non-metals is possible with imaging mass spectrometry. Here, cryo-slices of biological specimens can be imaged with laser ablation-ICP-MS; identification might be possible with ESI/MALDI of neighboring slices to provide more analytical information than merely the mapping of elements. However, the lack of reliable validated quantification strategies is the major problem coupled with bio-elemental imaging (Hare, Austin, & Doble, 2012).

3. *CE-ICP-MS*

Besides these two most-used configurations, other hyphenated techniques are also possible. Coupling of capillary electrophoresis (CE) to ICP-MS, for example, has been applied in analytical laboratories since the early 2000s (Kannamkumarath et al., 2002). At first, a certain stagnation occurred because the most critical point in coupling is the interface that must maintain a stable electrical circuit from CE to nebulizer (Michalke, 2005).

Also, because of low flow rate options, miniaturization of the nebulizer was necessary. In addition, several buffer components displayed detrimental effects in the nebulizer tip. Recently, however, this coupled procedure is more often used (though mostly in small molecule research), because enhanced commercial CE-ICP-MS interfaces became available (Schaumloffel & Prange, 1999; Yin, Li, & Yan, 2008; Liu et al., 2013) and more-appropriately chromatographic buffers were selected (Timerbaev et al., 2012).

We should mention that other coupled techniques, including GC-ICP-MS, exist, with great separation potential for volatile components. Here, the instrument settings are far different in comparison to normal wet plasma conditions. However, they are not used in the context of proteomic applications, and further discussion is thus out of the scope of this review.

III. PHOSPHOPROTEOMICS APPLICATIONS WITH MOLECULAR MS: CHALLENGES

Reversible protein phosphorylation works as a molecular switch that can change the function of proteins through alteration of the enzyme activity, to change the stability of a protein or vary how the protein interacts with other proteins in a very short time frame (Gafken & Lampe, 2006). This reversible phosphorylation event is controlled by the opposite interplay between large families of protein kinases and phosphatases (Kosako & Nagano, 2011). Deregulation of the phosphorylation event, and thus of kinase activity, is commonly associated with various cancers (Harsha & Pandey, 2010; Kettenbach & Gerber, 2011). In this regard, knowledge of the signaling pathways involved in malignancies has a potential to reveal mechanistic keystones of cellular transformation. A clear demonstration about how phosphoproteomics continues to play an important role to unravel complex signaling networks is given by Huang and White (Huang & White, 2008). Here, the authors illustrate that major contributions of phosphoproteomic techniques can elucidate the phosphorylation network present in disorders, such as DNA damage response signaling pathways.

To study the *in vivo* phosphorylation event, several different biochemical strategies can be applied such as labeling cells with radioactive phosphate or phosphospecific antibody arrays (Guo & Huang, 2013). With recent advances made in the MS-based phosphoproteomic field, it becomes clear that large-scale analysis of a complete phosphoproteome becomes more and more feasible. However, researchers are still confronted with several challenges (Mann et al., 2002; Nilsson, 2012). First, because stoichiometry of phosphorylation is quite low in general, only a small fraction of the protein is phosphorylated at a given time as a result of a stimulus. Second, detection of phosphopeptides is impaired by signal suppression due to the presence of more-abundant nonphosphorylated peptides in a proteomic digest. The presence of phosphopeptides within the large background of unphosphorylated peptides adds unwanted complexity to the experiment and therefore, phosphopeptides should be enriched prior to introduction into the mass spectrometer. Third, multiple phosphorylation sites can be present in one protein, to allow the protein to adapt several functions, depending on which phosphorylation site becomes occupied. This multitude of reaction sites also means that different phosphorylated forms of the same proteins can thus exist. Fourth, protein phosphorylation is a very dynamic process that constantly changes throughout the life of a cell. This dynamic regulation has thus high demands on the required analytical methods. Fifth, most analytical instruments have a limited dynamic range, which makes detection of minor phosphorylation sites difficult. Sixth, during sample preparation, phosphatases could dephosphorylate residues unless precautions are taken to inhibit their activity.

For several of these challenges, strategies for a more-efficient mass spectrometric detection have been developed. One of the most beneficial developments by far, is the variety of phosphopeptide enrichment strategies. These enrichment methods include immunoaffinity assays, in which antibodies raised against a protein or against a specific phosphosite, are used to enrich phosphopeptides or a certain subset of them. Enrichment methods based on metal-assisted affinity chromatography, such as immobilized metal affinity chromatography (IMAC) or metal-oxide materials (TiO_2) are applied even more often. Also, other chromatography-based methods, including ion exchange (strong cation exchange [SCX]) or hydrophilic interaction chromatography (HILIC), are applied in phosphoproteomics research. Several excellent reviews of all these phosphoprotein/peptide enrichment methods have been published (Mann et al., 2002; Thingholm, Jensen, & Larsen, 2009; Harsha & Pandey, 2010; Leitner, Sturm, & Linder, 2011; Engholm-Keller & Larsen, 2013).

Identification of phosphoproteins and characterization of different phosphorylation sites are usually performed with molecular mass spectrometry. Although enrichment techniques allow detection of phosphopeptides quite easily, phosphopeptide fragmentations must be improved to provide phosphopeptide characterization (Gafken & Lampe, 2006). For example, during collision-induced dissociation (CID), a fragmentation method implemented on most mass spectrometers, many pSer or pThr peptides exhibit neutral loss of phosphoric acid or a phosphate group and water, whereas for peptides phosphorylated at tyrosine, the modification mostly remains stable. In that regard, a scan for the neutral loss of H_3PO_4 (98 Da) and HPO_3 (80 Da) is mostly applied to detect phosphoSer- and phosphoThr-containing peptides whereas a precursor-ion scan to detect an immonium ion (in positive mode) at m/z 216.043 is characteristic for phosphoTyr-containing peptides. This phenomenon can lead to relatively uninformative MS/MS data, which can provide only limited information about the sequence of the peptide. Detection of the loss of PO_3 (79 Da) during precursor ion scanning under alkaline conditions seems to be the most-effective approach. Unfortunately, such basic experimental conditions are not easily compatible with HPLC (Pereira Navaza, Ruiz Encinar, & Sanz-Medel, 2007).

Also, using these MS/MS spectra, it is very difficult to one locate the site of phosphorylation within apeptide. Although newer fragmentation methods, including electron transfer dissociation (ETD), do not cause the loss of the labile phosphate group, they do not completely replace the more general CID, because these techniques have limitations such as slower scan speed and lower fragmentation efficiency. More details can be found in the recent excellent reviews on this topic (Thingholm, Jensen, & Larsen, 2009; Nilsson, 2012).

In cellular systems, changes in protein phosphorylation are a consequence of triggers or biological effects. Measurement of these phosphorylation changes is thus critical to understand the

biology of a phosphorylation event. In general phosphoproteomic approaches, the relative change in phosphoprotein levels between two cell states, is determined with isotopic or isobaric labeling approaches (Schreiber et al., 2008). Narumi and colleagues, for example, performed a large-scale phosphoproteomic experiment with isobaric tag for relative and absolute quantification (iTRAQ) with subsequent validation by targeted approaches like, for example, selected reaction monitoring (SRM) (Narumi et al., 2012). Although they were able to quantify 1927 phosphoproteins, only relative measurements could be obtained (Narumi et al., 2012). Several other research groups applied stable isotopic labeling of amino acids in cell cultures (SILAC) as a quantification method for phosphoproteomics experiments (Mann, 2006; Hilger et al., 2009; Oppermann et al., 2009). Also here, only relative quantification results are achieved.

For absolute quantification with molecular MS, the "AQUA" (absolute quantification) technique is preferred. AQUA allows sensitive and targeted quantitation of proteins and PTMs in complex protein mixtures with stable isotope labeled peptides as internal standards (Kettenbach & Gerber, 2011). A peptide of interest is synthesized and has a mass increase due to the incorporation of, for example, a single isotope labeled arginine ($^{13}C_6$,$^{15}N_4$) into the synthetic peptide, relative to the native peptide. Because the two forms have the same chromatographic and ionization characteristics, the mass spectrometry signal from the quantified synthetic peptide can be compared to the signal of the native peptide. This labeling schema will ultimately allow determination of the absolute quantity of the protein. For quantification of a phosphopeptide, the AQUA peptide is synthesized with a phosphoserine, phosphothreonine, and phosphotyrosine in addition to the isotopically labeled amino acid. When the phosphorylated and nonphosphorylated forms of an AQUA peptide are both used in a single experiment, the amount of the total protein and the ratio of phosphorylation at that site can both be determined simultaneously (Gafken & Lampe, 2006). However, for large-scale phosphoproteomics studies, this approach is not suited, because development of the AQUA peptides is time-consuming and costly. In that regard, the only method that can perform reliable absolute quantitation of phosphorylation without the need of isotopic labels is elemental mass spectrometry.

IV. DETECTION AND QUANTIFICATION OF PHOSPHOPROTEINS USING ICP-MS

Molecular mass spectrometry is well suited to study a phosphoproteome. However, absolute quantification of phosphopeptides remains a challenge. The use of ICP-MS in this context can be beneficial (Pereira Navaza, Ruiz Encinar, & Sanz-Medel, 2007). At first, analysis of the ^{31}P signal from phosphopeptides with ICP-MS seems to be impossible due to two physical facts: (1) the low ionization efficiency of phosphorus with the ICP source due to the high ionization potential of 10.484 eV. (2) the occurrence of severe polyatomic interferences from sample matrix components. However, as already mentioned, in recent years these issues are circumvented and several sophisticated solutions have been proposed. In the next section, we will critically discuss some important phosphopeptide-oriented ICP-MS studies carried out so far (Table 1) and give a clear overview of the potential of ICP-MS in phosphoproteomic research. As is clear from the several studies presented, the complementary use of molecular MS is still mandatory to elucidate amino acid sequences of phosphopeptides quantified with ICP-MS. This structural information is essential to translate the pmol of "^{31}P" quantified for each chromatographic peak in absolute pmol of phosphopeptide/protein and, of course, to elucidate the phosphorylation site(s) in a peptide (Pereira, Ruiz, & Sanz-Medel, 2007).

A. Detection of Phosphopeptides Using LC Separation

Lehmann's research group pioneered the use of elemental MS to detect phosphopeptides (Wind et al., 2001). They reported that detection of specific ^{31}P signals provided from ICP-MS resulted

TABLE 1. Overview of phosphoproteomics studies using ICP-MS.

Reference	*LC characteristics*	*Enrichment*	*Instruments used*	*flow rate*	*Modelpeptides*	*Combination with molecular MS*
Wind et al. 2001a	Cap LC ; Vydac C18 column	no	ICP-Q-MS (H_2 = carrier gas) ; SF-ICP-MS	4 µl/min	yes	yes
Wind et al. 2001b	Cap LC ; Vydac C18 column	no	SF-ICP-MS	4 µl/min	yes	yes
Profrock et al. 2005	CapLC and nanoLC ; Zorbax SB C18 column	no	ICP-Q-MS (He = collision gas)	4 µl/min or 300nl/min	yes	yes
Krüger et al. 2007	Cap LC ; Vydac C18 column	MOAC	SF-ICP-MS	2 µl/min	no	no
Navaza et al. 2007a	µLC; Zorbax SB C18 column	no	ICP-Q-MS (He = collision gas)	3.5 µl/min + sheath-flow 5.5µl/min)	yes	yes
Navaza et al. 2008a	CapLC; Zorbax SB C18 column	TiO2	ICP-Q-MS (He = collision gas)	3.5 µl/min + sheath-flow 5.5µl/min)	yes	yes
Ellis et al. 2008	CapLC; Zorbax SB C18 column	no	ICP-Q-MS (He = collision gas)	10µl/min	no	yes
Kroening et al. 2010	CapLC; Zorbax SB C18 column	no	ICP-Q-MS (He = collision gas)	5µl/min	no	yes
Pröfrock D. 2010	CapLC; Zorbax SB C18 column	no	ICP-Q-MS (He = collision gas)	4µl/min	yes	yes
Pröfrock and Prange 2009	CapLC; Zorbax SB C18 column	no	ICP-Q-MS (He = collision gas)	2µl/min + sheath-flow 2µl/min	yes	yes
Zinn et al. 2009	CapLC; Waters C18 column	no	SF-ICP-MS	4.6 µl/min	yes	yes
Diez et al. 2012	CapLC; Zorbax SB C18 column	no	ICP-QQQ-MS (O_2 = reaction gas)	5 µl/min + sheath-flow 4.5µl/min)	yes	no

Reference	*electrophoresis characteristics*	*Enrichment*	*Instruments used*	*Modelpeptides*	*Combination with molecular MS*
Marshall et al. 2002	1D PAGE + electroblotting PVDF	no	ICP-Q-MS (He/ H_2 = collision gas)	yes	no
Bandura et al. 2004	1D PAGE + drying	no	ICP-Q-MS (O_2 = reaction gas)	no	yes

in much simpler chromatograms than those obtained with UV or ESI-MS. With tryptic digests of model peptides, including beta-casein, they introduced phosphorus (^{31}P) detection with either Q-ICP-MS or SF-ICP-MS into the field of phosphoproteomics (Wind et al., 2001). In the same year, they also introduced a new approach for the accurate determination of the degree of phosphorylation in proteins and peptides. Because the ICP response of phosphorus is dependent on the solvent composition (high amounts of organic solvents will introduce first signal enhancement and finally signal suppression) (Grindlay et al., 2008), and LC separation is mostly based on different concentrations of organic solvents, a correction factor was determined to minimize this effect. The same authors proposed the use of sulfur as a key element for absolute quantification of peptides and proteins (Wind et al., 2003). Sulfur is present in two naturally occurring amino acids, cysteine and methionine and therefore, in the majority of all proteins (>98%). In peptides, however, sulfur-containing amino acids are less omni-present because 26.6% of tryptic peptides contain at least one cysteine and 25.5% have at least a methionine in their amino acid sequence (Prange & Proefrock, 2008). Therefore, ^{31}P chromatogram peaks indicate elution of phosphopeptides, whereas ^{34}S chromatogram signals represent peptides with cysteine, methionine or both. This "sulfur" signal can be used as a "probe" to quantify both phosphorus and dephosphorus forms. Integration of all peaks on the respective traces gives access to a P/S ratio that represents protein phosphorylation stoichiometry. Because the signal varies slightly as a function of the mobile phase composition, corrections against a phosphorus/sulfur standard with known concentration must be integrated. This corrected molar P/S ratio can afterwards be converted into the degree of phosphorylation, if the amino acid sequence is obtained. The quantitative determination of the majority of proteins on the basis of their natural sulfur content becomes possible. However, complementary application of ESI- or MALDI-based MS approaches is still necessary to further clarify stoichiometric and identification issues. We should point out, though, that in case of complex peptide mixtures, a superb isolation, for example, three-dimensional LC, is still necessary because the coelution of numerous S-peptides or even multiple sulfur-containing residues in amino acid sequence could result in a very complex chromatograms and thus challenging, and even misleading, interpretation.

In 2005, Pröfrock and co-workers developed a new interface that allowed an efficient coupling of capLC and nano-LC to ICP-MS instruments (Pröfrock et al., 2005). Although Wind et al. (2001) already used a total consumption nebulizer, further developments were necessary to allow a reliable integration of low flow LC (~4 μl/min or lower) with ICP-MS. The authors could show that their optimized configuration could successfully be applied to phosphorylation studies with a tryptic digest of beta casein as model protein (Pröfrock et al., 2005).

Although several developments were made in the field, it took until 2007 before the first manuscript that described more complex samples for phospho screening was published. Krüger and colleagues were one of the first groups who implemented the P/S ratio rule of Wind et al., in order to find a global protein phosphorylation level in *Arabidopsis thaliana* plants (Krüger et al., 2007). To obtain an idea about the "global" phosphorylation state, phosphopeptides were enriched with metal-oxide affinity chromatography (MOAC) before analysis by LC-ICP-MS. They found varying levels of protein phosphorylation in samples from different developmental stages that indicated that phosphorus-based signaling pathways are also important in plant development (Krüger et al., 2007). Furthermore, the same research group also studied the protein and proteome phosphorylation stoichiometry of bacterial cells and eukaryotic cells with micro LC-ICP-MS and LA-ICP-MS (Krüger et al., 2006).

Sanz-Medel's research group implemented bis-4-nitrophenyl-phosphate (BNPP) as compound-independent phosphorus standard to allow accurate absolute phosphopeptide quantification, even in complex peptide mixtures. The implementation of this technique did remove some issues of P/S ratio applications because this technique can only be applied to cysteine- and methionine-containing peptides, which represent 30% of the peptide population. To minimize the changes throughout the reverse phase gradient, they also developed a post-column sheath-flow system to ensure that the signal fluctuates less with separation gradients (Pereira, Ruiz, & Sanz-Medel, 2007). In this way, sensitivity remained essentially constant along the reverse phase gradient, and the response factor obtained for BNPP (area/ng P injected) could be applied to quantify different phosphopeptides. More recently, another research group introduced a more complex system to compensate for the gradient related effects. This system compensates sensitivity changes at the very low or very high acetonitrile concentrations with two capLC systems with inverse gradients (Pröfrock & Prange, 2009).

An other publication from the Sanz-Medel research group showed that phosphopeptide enrichment with a TiO_2 cartridge procedure can be reproducibly applied. However, during enrichment, recoveries obtained from the cartridges were quite low and species-dependent; that factor must be taken into account in absolute quantifications (Navaza et al., 2008a). Soon afterwards, the same group assessed the great potential of capillary HPLC coupled to ICP-MS to study peptide phosphorylation dynamics (Navaza, Encinar, & Sanz-Medel, 2008b). The high precision associated with absolute quantification of the phosphopeptides (1–4% RSD) allowed a clear discrimination among 15 different phosphorylation levels along its long dephosphorylation process, and demonstrated the high discrimination potential among very-close phosphorylation degrees (Fig. 2). This powerful approach is fully complementary to the MS global phosphorylation dynamic studies with isotopic labeling (Olsen et al., 2006). After identification of the phosphorylation dynamics of a protein as a key parameter in the response of a cell to a specific stimulus with MS isotopic encoding techniques, a deeper insight into the temporal involvement of such protein in the whole signaling process could be studied in greater detail after proper isolation, preconcentration and analysis by LC-ICP-MS.

In the next years, several research articles, which studied a phosphoproteome with LC-ICP-MS, were published. Ellis et al. (2008), for example, studied protein phosphorylation in a specific fraction of human cerebral spinal fluid. They used capLC-ICP-MS to screen ^{31}P, and combined these results with ion-trap mass spectrometry for peptide identification and phosphosite characterization. A more complex fraction was also analyzed (Kroening et al., 2010). They showed that the parallel use of molecular and elemental MS has potential for biomarker development. ICP-MS allows a simplified screening among different patients, to enable subdivision of the samples before

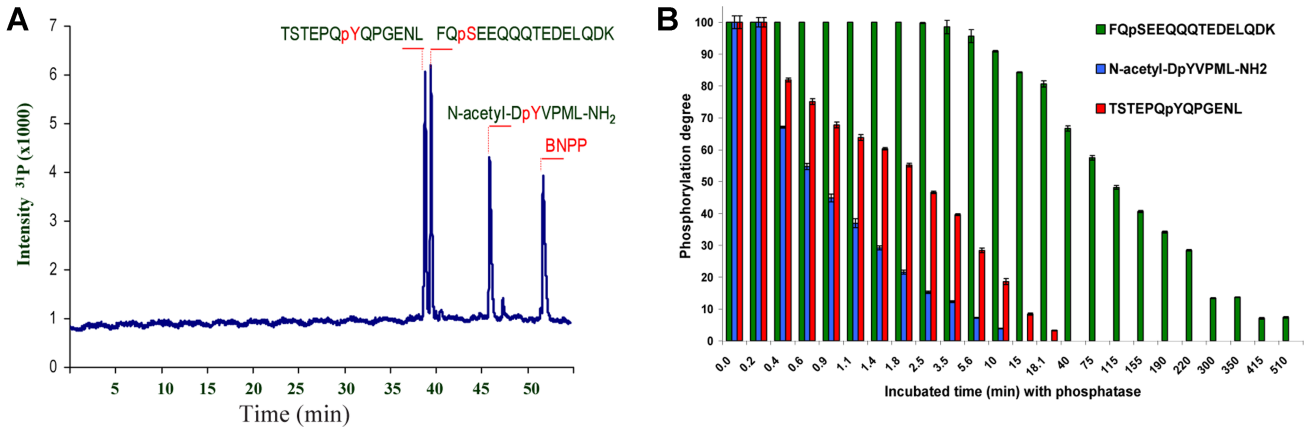

FIGURE 2. (**A**) Reverse phase capHPLC–ICP-MS chromatogram of a mixture of three synthetic phosphorylated peptides before starting the dephosphorylation treatment with alkaline phosphates. (**B**) Time course quantitative data corresponding to the phosphorylation degree (%) of the three peptides along the dephosphorylation process.

more-extensive experiments are performed (Kroening et al., 2010).

Besides the complementary use of ESI-source MS, MALDI-TOF-TOF is also applied in research applications that combine LC-ICP-MS with molecular MS. Pröfrock an co-workers were the first to develop a novel combination that allowed LC-ICP-MS and parallel online micro fractionation collection for MALDI-TOF-TOF experiments in phosphoproteomics applications. With beta-casein as a model protein, the author also mentioned that the approach is currently limited for analysis of single purified proteins, because better fractionation should be established before application to complex samples (Pröfrock, 2010).

Later, Lehmann's research group introduced the PASTA peptide concept. These phosphorus-based absolutely quantified standard (PASTA) peptides are a novel tool to prepare absolutely quantified stable isotope labeled peptide solutions for analysis with molecular MS. With capLC-(ICP + ESI) − MS, synthetic phosphopeptides could be absolutely quantified with ICP-MS, with BNPP as internal standard, and the identification of the peak could be confirmed with ESI-MS. Afterwards, such phosphopeptides could be subjected to complete dephosphorylation to obtain corresponding stable isotope labeled unphosphorylated peptides, which can be employed as standards in subsequent, more-conventional quantitative analysis with LC-ESI-MS (Zinn et al., 2009). Although this approach is fairly unknown, it clearly demonstrates the potential of ICP-MS in quantitative proteomic research.

Very recently, Diez et al. (2012) introduced triple quad ICP-MS (ICP-QQQ) into proteomics and phosphoproteomics. The first quadrupole can be operated as a mass filter to select target analyte ions (^{31}P, ^{32}S, and their corresponding polyatomic interferences). Therefore, only selected ions could enter the cell and react with O_2, to reduce the interferences produced by matrix ions as well as background noise. After optimization of the cell conditions, product ions formed for the targets, phosphorus ($^{47}PO^+$) and sulfur ($^{48}SO^+$), could be detected with enhanced selectivity and sensitivity (low fmol level). It is expected that the combination of LC to these instruments will boost ICP-MS applications in proteomics and phosphoproteomics research in the near future.

B. Detection of Phosphopeptides With Laser Ablation

In complex samples, high-resolution protein separation can also, besides liquid chromatography, be achieved with gel electrophoresis. Coupling of gel electrophoresis with ICP-MS was established with laser ablation more than 10 years ago.

Marshall et al. (2002) were the first to report the use of laser ablation to detect and quantify phosphoproteins after gel electrophoresis. They stated that ablation from gels is problematic due to high background values. However, they nicely showed that phosphorous-related analysis is possible when the gel is blotted onto a membrane. Phosphopeptides of different concentration deposited onto membranes (dot blots) were subsequently analyzed with LA-ICP-MS. In the ICP output, clear differences between phosphopeptide concentrations were apparent (Marshall et al., 2002).

In 2004, Bandura and co-workers used laser ablation to detect differences in total phosphorus content (Bandura, Ornatsky, & Liao, 2004). A 1D gel was not blotted onto membranes, but "dried" between two cellophane sheets to obtain a vacuum dry gel from which protein spots were ablated. Using this approach, the authors could show that phosphorus was present in higher amounts in human colon cancer tissues compared to their paired "normal" counterpart, to indicate differences in the activity of kinases. Moreover, they attained sub-nanomole amounts of phosphoprotein detection, indicating that this technique has promise in future cancer research projects.

The application of 2D gel electrophoresis and LA-ICP-MS to detect metallo- and phosphoproteins was extensively used by the research groups of Sabine Becker and Michael Przybylski (Becker et al., 2005b, 2008). With a parallel approach between LA-ICP-MS and MALDI-FTICR-MS, they first established the protocol with model peptides such as, for example, the highly phosphorylated tau protein (Becker et al., 2003). In a more advanced study, human brain proteins were separated with 2D PAGE. Next, 176 well-separated protein spots were screened for

the presence of P (and other elements, including Cu, Zn, and U) in just a few hours, to detect P-containing proteins (Becker et al., 2005a).

V. FUTURE PERSPECTIVES

Although not often applied in regular phosphoproteomic approaches, this review has shown that ICP-MS is a complementary MS technique that allows fast phosphopeptide screening and absolute quantification of individual phosphopeptides. Unfortunately, a comprehensive detection of individual phosphoproteins/peptides in a complex (biological) sample remains still challenging as it requires high-resolution protein separation. In this regard, an extended fractionation might be possible to obtain individual peptides; however, the question remains whether ICP-MS technologies will ever be able to detect these ultra-low phosphopeptide concentrations. New ICP-MS instrumental developments, such as the ICP-QQQ that can offer very low detection limits for P (and S), are expected to face this challenge.

In future applications, we therefore expect that more targeted phosphoproteomic approaches will be pursued. The use of stable isotope labeled standard (SIS) peptide analogues in combination with targeted MS-based approaches such as multiple reaction monitoring (MRM), have already been show to allow absolute quantification of peptides in complex matrices (Kuzyk et al., 2009). With this strategy, determination of phosphorylation stoichiometry is also feasible (Domanski, Murphy, & Borchers, 2010). Of course the use of ICP-MS would help in the quantitative characterization of such SIS standards. Another approach that allows absolute quantification of targeted peptides includes the production of phosphorus-based absolutely quantified standard (PASTA) peptides. With this strategy, the absolute quantification of peptide phosphorylation is possible when peak areas of peptides of interest are compared to the corresponding PASTA peptide signal, resulting in their molar ratio (Zinn et al., 2009).

Finally, some interesting applications of ICP-MS in phosphoproteomics have been already demonstrated and others can be easily foreseen. Among them, certification of protein/phosphoprotein standards, kinetic studies of targeted phosphoproteins and quantitative assessment of sample preparation steps stand out. Exploration of all of these applications towards more complex samples will provide a new level of information on absolute phosphoprotein quantitation.

ACKNOWLEDGMENTS

The authors acknowledge the Flemish Institute for Technological Research (VITO). Evelyne Maes is funded by a Stichting Emmanuel van der Schueren research grant of the Vlaamse Liga tegen Kanker (VLK).

REFERENCES

Aebersold R, Bader GD, Edwards AM, van Eyk JE, Kussmann M, Qin J, Omenn GS. 2013. The biology/disease-driven human proteome project (B/D-HPP): Enabling protein research for the life sciences community. J Proteome Res 12:23–27.

Bandura DR, Ornatsky O, Liao L. 2004. Characterization of phosphorus content of biological samples by ICP-DRC-MS: Potential tool for cancer research. J Anal At Spectrom 19:96–100.

Becker JS, Becker JS. 2010. Imaging of metals, metalloids, and non-metals by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS) in biological tissues. Methods Mol Biol 656:51–82.

Becker JS, Becker JS, Zoriy MV, Dobrowolska J, Matucsh A. 2007. Imaging mass spectrometry in biological tissues by laser ablation inductively coupled plasma mass spectrometry. Eur J Mass Spectrom (Chichester, Eng) 13:1–6.

Becker JS, Boulyga SF, Pickhardt C, Becker J, Buddrus S, Przybylski M. 2003. Determination of phosphorus in small amounts of protein samples by ICP-MS. Anal Bioanal Chem 375:561–566.

Becker JS, Dietze HJ. 1998. Ultratrace and precise isotope analysis by double-focusing sector field inductively coupled plasma mass spectrometry. J Anal At Spectrom 13:1057–1063.

Becker JS, Jakubowski N. 2009. The synergy of elemental and biomolecular mass spectrometry: New analytical strategies in life sciences. Chem Soc Rev 38:1969–1983.

Becker JS, Mounicou S, Zoriy MV, Becker JS, Lobinski R. 2008. Analysis of metal-binding proteins separated by non-denaturating gel electrophoresis using matrix-assisted laser desorption/ionization mass spectrometry (MALDI-MS) and laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). Talanta 76:1183–1188.

Becker JS, Zoriy M, Becker JS, Pickhardt C, Damoc E, Juhacz G, Palkovits M, Przybylski M. 2005a. Determination of phosphorus-, copper-, and zinc-containing human brain proteins by LA-ICPMS and MALDI-FTICR-MS. Anal Chem 77:5851–5860.

Becker JS, Zoriy M, Matusch A, Wu B, Salber D, Palm C, Becker JS. 2010. Bioimaging of metals by laser ablation inductively coupled plasma mass spectrometry (LA-ICP-MS). Mass Spectrom Rev 29: 156–175.

Becker JS, Zoriy MV, Pickhardt C, Palomero-Gallagher N, Zilles K. 2005b. Imaging of copper, zinc, and other elements in thin section of human brain samples (hippocampus) by laser ablation inductively coupled plasma mass spectrometry. Anal Chem 77:3208–3216.

Bettmer J, Montes BM, Encinar JR, Fernandez Sanchez ML, Fernandez de la Campa Mdel R, Sanz Medel A. 2009. The emerging role of ICP-MS in proteomic analysis. J Proteomics 72:989–1005.

Diez FS, Sugishama N, Ruiz EJ, Sanz-Medel A. 2012. Triple quad ICPMS (ICPQQQ) as a new tool for absolute quantitative proteomics and phosphoproteomics. Anal Chem 84:5851–5857.

Domanski D, Murphy LC, Borchers CH. 2010. Assay development for the determination of phosphorylation stoichiometry using multiple reaction monitoring methods with and without phosphatase treatment: Application to breast cancer signaling pathways. Anal Chem 82:5610–5620.

Ellis J, Grimm R, Clark JF, Pyne-Gaithman G, Wilbur S, Caruso JA. 2008. Studying protein phosphorylation in low MW CSF fractions with capLC-ICPMS and nanoLC-CHIP-ITMS for identification of phosphoproteins. J Proteome Res 7:4736–4742.

Engholm-Keller K, Larsen MR. 2013. Technologies and challenges in large-scale phosphoproteomics. Proteomics 13:910–931.

Gafken PR, Lampe PD. 2006. Methodologies for characterizing phosphoproteins by mass spectrometry. Cell Commun Adhes 13:249–262.

Grindlay G, Gras L, Mora J, de Loos-Vollebregt M. 2008. Carbon-related matrix effects in inductively coupled plasma atomic emission spectrometry. Spectrochim Acta A Mol Biomol Spectrosc 63:234–243.

Guo M, Huang BX. 2013. Integration of phosphoproteomic, chemical, and biological strategies for the functional analysis of targeted protein phosphorylation. Proteomics 13:424–437.

Hare D, Austin C, Doble P. 2012. Quantification strategies for elemental imaging of biological samples using laser ablation-inductively coupled plasma-mass spectrometry. Analyst 137:1527–1537.

Harsha HC, Pandey A. 2010. Phosphoproteomics in cancer. Mol Oncol 4:482–495.

Hilger M, Bonaldi T, Gnad F, Mann M. 2009. Systems-wide analysis of a phosphatase knock-down by quantitative proteomics and phosphoproteomics. Mol Cell Proteomics 8:1908–1920.

Huang PH, White FM. 2008. Phosphoproteomics: Unraveling the signaling web. Mol Cell 31:777–781.

Kannamkumarath SS, Wrobel K, Wrobel K, B'Hymer C, Caruso JA. 2002. Capillary electrophoresis-inductively coupled plasma-mass spectrometry: An attractive complementary technique for elemental speciation analysis. J Chromatogr A 975:245–266.

Kettenbach AN, Gerber SA. 2011. Rapid and reproducible single-stage phosphopeptide enrichment of complex peptide mixtures: Application to general and phosphotyrosine-specific phosphoproteomics experiments. Anal Chem 83:7635–7644.

Konz I, Fernandez B, Fernandez ML, Pereiro R, Sanz-Medel A. 2012. Laser ablation ICP-MS for quantitative biomedical applications. Anal Bioanal Chem 403:2113–2125.

Kosako H, Nagano K. 2011. Quantitative phosphoproteomics strategies for understanding protein kinase-mediated signal transduction pathways. Expert Rev Proteomics 8:81–94.

Kroening KK, Kuhlmann J, Easter R, Clark JF, Pyne-Geithman G, Caruso JA. 2010. Protein phosphorylation studies of cerebral spinal fluid for potential biomarker development. Metallomics 2:334–341.

Krüger R, Kubler D, Pallisse R, Burkovski A, Lehmann WD. 2006. Protein and proteome phosphorylation stoichiometry analysis by element mass spectrometry. Anal Chem 78:1987–1994.

Krüger R, Wolschin F, Weckwerth W, Bettmer J, Lehmann WD. 2007. Plant protein phosphorylation monitored by capillary liquid chromatography—Element mass spectrometry. Biochem Biophys Res Commun 355:89–96.

Kuzyk MA, Smith D, Yang J, Cross TJ, Jackson AM, Hardie DB, Anderson NL, Borchers CH. 2009. Multiple reaction monitoring-based, multiplexed, absolute quantitation of 45 proteins in human plasma. Mol Cell Proteomics 8:1860–1877.

Leitner A, Sturm M, Lindner W. 2011. Tools for analyzing the phosphoproteome and other phosphorylated biomolecules: A review. Anal Chim Acta 703:19–30.

Liu L, He B, Yun Z, Sun J, Jiang G. 2013. Speciation analysis of arsenic compounds by capillary electrophoresis on-line coupled with inductively coupled plasma mass spectrometry using a novel interface. J Chromatogr A 1304:227–233.

Lobinski R, Schaumloffel D, Szpunar J. 2006. Mass spectrometry in bioinorganic analytical chemistry. Mass Spectrom Rev 25:255–289.

Mann M. 2006. Functional and quantitative proteomics using SILAC. Nat Rev Mol Cell Biol 7:952–958.

Mann M, Ong SE, Gronborg M, Steen H, Jensen ON, Pandey A. 2002. Analysis of protein phosphorylation using mass spectrometry: Deciphering the phosphoproteome. Trends Biotechnol 20:261–268.

Marshall P, Heudi O, Bains S, Freeman HN, Abou-Shakra F, Reardon K. 2002. The determination of protein phosphorylation on electrophoresis gel blots by laser ablation inductively coupled plasma-mass spectrometry. Analyst 127:459–461.

Michalke B. 2005. Capillary electrophoresis-inductively coupled plasma-mass spectrometry: A report on technical principles and problem solutions, potential, and limitations of this technology as well as on examples of application. Electrophoresis 26:1584–1597.

Moldovan M, Krupp EM, Holliday AE, Donard OFX. 2004. High resolution sector field ICP-MS and multicollector ICP-MS as tools for trace metal speciation in environmental studies: A review. J Anal At. Spectrom 19: 815–822.

Montes-Bayon M, DeNicola K, Caruso JA. 2003. Liquid chromatography-inductively coupled plasma mass spectrometry. J Chromatogr A 1000: 457–476.

Mounicou S, Szpunar J, Lobinski R. 2010. Inductively-coupled plasma mass spectrometry in proteomics, metabolomics and metallomics studies. Eur J Mass Spectrom (Chichester, Eng) 16:243–253.

Narumi R, Murakami T, Kuga T, Adachi J, Shiromizu T, Muraoka S, Kume H, Kodera Y, Matsumoto M, Nakayama K, Miyamoto Y, Ishitobi M, Inaji H, Kato K, Tomonaga T. 2012. A strategy for large-scale phosphoproteomics and SRM-based validation of human breast cancer tissue samples. J Proteome Res 11:5311–5322.

Navaza AP, Encinar JR, et al. 2007a. Absolute and accurate quantification of protein phosphorylation by using an elemental phosphorus standard and element mass spectrometry. Angew Chem Int Ed Engl 46:569–571.

Navaza AP, Encinar JR, et al. 2007b. Quantitative protein phosphorylation analysis: The role of ICP-MS. J Anal At Spectrom 22:1223–1237.

Navaza AP, Encinar JR, Carrascal M, Abian J, Sanz-Medel A. 2008a. Absolute and site-specific quantification of protein phosphorylation using integrated elemental and molecular mass spectrometry: Its potential to assess phosphopeptide enrichment procedures. Anal Chem 80:1777–1787.

Navaza AP, Encinar JR, Sanz-Medel A. 2008b. Element mass spectrometry as a tool for high-resolution temporal dynamics investigations of peptide phosphorylation. Chem Commun 6230–6232.

Nilsson CL. 2012. Advances in quantitative phosphoproteomics. Anal Chem 84:735–746.

Olsen JV, Blagoev B, Gnad F, Macek B, Kumar C, Mortensen P, Mann M. 2006. Global, in vivo, and site-specific phosphorylation dynamics in signaling networks. Cell 127:635–648.

Oppermann FS, Gnad F, Olsen JV, Hornberger R, Greff Z, Keri G, Mann M, Daub H. 2009. Large-scale proteomics analysis of the human kinome. Mol Cell Proteomics 8:1751–1764.

Paradela A, Albar JP. 2008. Advances in the analysis of protein phosphorylation. J Proteome Res 7:1809–1818.

Prange A, Proefrock D. 2008. Chemical labels and natural element tags for the quantitative analysis of bio-molecules. J Anal At Spectrom 23:432–459.

Pröfrock D. 2010. Progress and possible applications of miniaturised separation techniques and elemental mass spectrometry for quantitative, heteroatom-tagged proteomics. Anal Bioanal Chem 398:2383–2401.

Pröfrock D, Leonhard P, Ruck W, Prange A. 2005. Development and characterisation of a new interface for coupling capillary LC with collision-cell ICP-MS and its application for phosphorylation profiling of tryptic protein digests. Anal Bioanal Chem 381:194–204.

Pröfrock D, Prange A. 2009. Compensation of gradient related effects when using capillary liquid chromatography and inductively coupled plasma mass spectrometry for the absolute quantification of phosphorylated peptides. J Chromatogr A 1216:6706–6715.

Pröfrock D, Prange A. 2012. Inductively coupled plasma-mass spectrometry (ICP-MS) for quantitative analysis in environmental and life sciences: A review of challenges, solutions, and trends. Appl Spectrosc 66: 843–868.

Ray SJ, Andrade F, Gamez G, McClenathan D, Rogers D, Schilling G, Wetzel W, Hieftje GM. 2004. Plasma-source mass spectrometry for speciation analysis: State-of-the-art. J Chromatogr A 1050:3–34.

Roux PP, Thibault P. 2013. The coming of age of phosphoproteomics; from large data sets to inference of protein functions. Mol Cell Proteomics 12:3453–3464.

Sanz-Medel A. 2010. ICP-MS for multiplex absolute determinations of proteins. Anal Bioanal Chem 398:1853–1859.

Sanz-Medel A, Montes-Bayón M, del Rosario Fernández de la Campa M, Encinar JR, Bettmer J. 2008. Elemental mass spectrometry for quantitative proteomics. Anal Bioanal Chem 390:3–16.

Sanz-Medel A, Montes-Bayon M, Luisa Fernandez SM. 2003. Trace element speciation by ICP-MS in large biomolecules and its potential for proteomics. Anal Bioanal Chem 377:236–247.

Schaumloffel D. 2007. New ways in qualitative and quantitative protein analysis: Nano chromatography coupled to element mass spectrometry. J Trace Elem Med Biol 21:18–22.

Schaumloffel D, Prange A. 1999. A new interface for combining capillary electrophoresis with inductively coupled plasma-mass spectrometry. Fresenius J Anal Chem 364:452–456.

Schaumloffel D, Ruiz EJ, Lobinski R. 2003. Development of a sheathless interface between reversed-phase capillary HPLC and ICPMS via a microflow total consumption nebulizer for selenopeptide mapping. Anal Chem 75:6837–6842.

Schreiber TB, Mausbacher N, Breitkopf SB, Grundner-Culemann K, Daub H. 2008. Quantitative phosphoproteomics—An emerging key technology in signal-transduction research. Proteomics 8:4416–4432.

Siethoff C, Feldmann I, Jakubowski N, Linscheid M. 1999. Quantitative determination of DNA adducts using liquid chromatography/electrospray ionization mass spectrometry and liquid chromatography/high-resolution inductively coupled plasma mass spectrometry. J Mass Spectrom 34:421–426.

Stefanka Z, Koellensperger G, Stingeder G, Hann S. 2006. Down-scaling narrowbore LC-ICP-MS to capillary LC-ICP-MS: A comparative study of different introduction systems. J Anal At Spectrom 21:86–89.

Svantesson E, Pettersson J, Markides KE. 2002. The use of inorganic elemental standards in the quantification of proteins and biomolecular compounds by inductively coupled plasma spectrometry. J Anal At Spectrom 17:491–496.

Szpunar J, Lobinski R, Prange A. 2003. Hyphenated techniques for elemental speciation in biological systems. Appl Spectrosc 57:102A–112A.

Templeton DM, Ariese F, Cornelis R, Danielsson LG, Muntau H, Van Leeuwen HP, Lobinski R. 2000. Guidelines for terms related to chemical speciation and fractionation of elements. Definitions, structural aspects, and methodological approaches (IUPAC Recommendations 2000). Pure Appl Chem 72:1453–1470.

Thingholm TE, Jensen ON, Larsen MR. 2009. Analytical strategies for phosphoproteomics. Proteomics 9:1451–1468.

Timerbaev AR, Pawlak K, Aleksenko SS, Foteeva LS, Matczuk M, Jarosz M. 2012. Advances of CE-ICP-MS in speciation analysis related to metalloproteomics of anticancer drugs. Talanta 102:164–170.

Wang M, Feng WY, Zhao YL, Chai ZF. 2010. ICP-MS-based strategies for protein quantification. Mass Spectrom Rev 29:326–348.

Wind M, Edler M, Jakubowski N, Linscheid M, Wesch H, Lehmann WD. 2001a. Analysis of protein phosphorylation by capillary liquid chromatography coupled to element mass spectrometry with 31P detection and to electrospray mass spectrometry. Anal Chem 73: 29–35.

Wind M, Wegener A, Eisenmenger A, Kellner R, Lehmann WD. 2003. Sulfur as the key element for quantitative protein analysis by capillary liquid chromatography coupled to element mass spectrometry. Angew Chem Int Ed Engl 42:3425–3427.

Wind M, Wesch H, Lehmann WD. 2001b. Protein phosphorylation degree: Determination by capillary liquid chromatography and inductively coupled plasma mass spectrometry. Anal Chem 73:3006–3010.

Yin XB, Li Y, Yan XP. 2008. CE-ICP-MS for studying interactions between metals and biomolecules. TrAC Trends Anal Chem 27:554–565.

Zhang Y, Fonslow BR, Shan B, Baek MC, Yates JR III. 2013. Protein analysis by shotgun/bottom-up proteomics. Chem Rev 113:2343–2394.

Zhao Y, Jensen ON. 2009. Modification-specific proteomics: Strategies for characterization of post-translational modifications using enrichment techniques. Proteomics 9:4632–4641.

Zinn N, Hahn B, Pipkorn R, Schwarzer D, Lehmann WD. 2009. Phosphorus-based absolutely quantified standard peptides for quantitative proteomics. J Proteome Res 8:4870–4875.